Cloning and characterization of a *Streptomyces antibioticus* ATCC11891 cyclophilin related to Gram negative bacteria cyclophilins☆

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Abstract Cyclophilins are folding helper enzymes and represent a family of the enzyme class of peptidyl-prolyl *cis–trans* isomerases. Here, we report the molecular cloning and biochemical characterization of *San*Cyp18, an 18-kDa cyclophilin from *Streptomyces antibioticus* ATCC11891 located in the cytoplasm and constitutively expressed during development. Amino acid sequence analysis revealed a much higher homology to cyclophilins from Gram negative bacteria than to known cyclophilins from *Streptomyces* or other Gram positive bacteria. *San*Cyp18 is inhibited weakly by CsA, with a K_i value of 21 μM, similar to cyclophilins from Gram negative bacteria. However, this value is more than 20-fold higher than the K_i values reported for cyclophilins from other Gram positive bacteria, which makes *San*Cyp18 unique within this group. The presence of *San*Cyp18 in *Streptomyces* is likely due to horizontal gene transmission from Gram-negative bacteria to *Streptomyces*.

Keywords: *San*Cyp18; PPIase; Cyclophilin; Cyclosporine A; *Streptomyces*

1. Introduction

Protein folding is thought to be a spontaneous process that transforms the linear primary amino acid sequence of the nascent polypeptide chain into a specific three-dimensional structure. Amino acids are mainly connected in proteins via the *trans* peptide bond. The propensity for a *cis* peptide bond to exist in native proteins is <0.1%. However, in the case of the peptidyl-prolyl bond, the *cis* conformation increases from 5% to 6% in the *native state* [1]. Refolding experiments of denaturated proteins showed that the *cis*/*trans* isomerization of peptidyl-prolyl bonds is often a rate-limiting process during protein folding events [2]. Peptidyl-prolyl *cis*/*trans* isomerases (PPIases; EC 5.2.1.8) accelerate the formation of the *cis*/*trans* equilibrium of peptidyl-prolyl bonds. Currently, the PPIase enzyme class includes three families: cyclophilins, FKBP (FK506 binding proteins), and parvulins [for reviews see [3–5]]. No sequence homology exists between the three families, which exhibit characteristic substrate specificities [6]. They can be distinguished with specific, natural product inhibitors: cyclophilins are inhibited by cyclosporine A (CsA), FKBPs by FK506 and rapamycin and parvulins by juglone [3].

Cyclophilins are ubiquitously distributed proteins, highly conserved during evolution and present in almost all organisms analyzed to date, with particular exceptions in bacteria such as *Mycoplasma genitalium* and some archaea [3]. They are widely expressed in many tissues and cellular compartments and, like the other PPIases, are relevant in cells under stress conditions in which protein folding is a limiting process [7–10]. More than 28 cyclophilins are encoded in the human genome, ranging from 18 to 165 kDa in molecular weight [11,12]. Although many of the eukaryotic cyclophilins have been studied extensively, few examples of functional characterization of these enzymes exist in prokaryotes, except *Escherichia coli* [13–18], *Bacillus subtilis* [19–22], *Streptomyces chrysomallus* [23,24], *Acinetobacter calcoaceticus* [25], *Legionella pneumophila* [26], *Erwinia chrysanthemi* [27] and *Halobacterium cutirubrum* [28]. The most obvious characteristic of bacterial cyclophilins is a lower affinity for CsA compared to human cyclophilin 18 (hCyp18). Cyclophilins from Gram negative bacteria have been shown to be particularly resistant to CsA [29].

Streptomyces is a mycelial bacterium whose natural habitat is the soil, where it carries out a notable degradative activity on organic substrates. Its developmental cycle is highly complex and involves programmed cell-death processes that are central to the differentiating cycle of the bacterium [30,31]. The signals that induce these phenomena are unknown, though stress conditions are known to induce these processes [A. Manteca and J. Sanchez, unpublished data and [32]]. PPIases may possibly be relevant in these processes and thus *Streptomyces* constitutes a good model to test the biological roles of these enzymes in bacteria. In a previous work [33], we described an 18-kDa cyclophilin from *S. antibioticus* which was proposed to have both PPIase and nuclease activity in vitro, being reminiscent of other reported cyclophilins with dual PPIase and nuclease activity [34]. In this paper, we report the cloning and extensive characterization of the *S. antibioticus* cyclophilin.

☆ The sequence of *San*Cyp18 is available at the GenBank Nucleotide Sequence Database under Accession No. AY343890.

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Abbreviations: CD, circular dichroism; CsA, cyclosporine A; HGT, horizontal gene transmission; ITC, isothermal titration calorimetry; K_a, association constant; K_d, dissociation constant; *M*r, molecular mass; NH-Np, 4-nitroanilide; PPIase, peptidyl-prolyl *cis–trans*isomerase; *San*Cyp18, *Streptomyces antibioticus* ATCC11891 cyclophilin

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The protein is located in the cytoplasm, is constitutively expressed and shows genetic and biochemical characteristics more similar to cyclophilins from Gram negative bacteria than to other *Streptomyces* cyclophilins and cyclophilins from other Gram positive bacteria. The *San*Cyp18 gene would have been acquired from the former group by horizontal gene transfer.

2. Materials and methods

2.1. Growth conditions

Streptomyces antibioticus ATCC11891 was cultured on solid or liquid GAE medium [35] at 30 °C and 200 rpm in the case of submerged conditions. Plates and liquid cultures were directly inoculated with 100 μl of a spore suspension (1.5×10^7 viable spores/ml). GAE medium plates were covered with sterile cellophane disks, before incubation [36].

2.2. DNA techniques

Chromosomal and plasmid DNA were isolated using standard procedures [37]. Separated DNA fragments were isolated from agarose gels using the QUIAEX IIR Gel Extraction System (Qiagen) and ligated into suitable vector molecules. *E. coli* cells were transformed by the $CaCl_2$ method [37].

2.3. Cloning and sequencing of streptomyces antibioticus cyclophilin gene

Two degenerated oligonucleotides corresponding to the amino acid sequence SAGRIV (5′-TCSGCSGGSCGSATCGT-3′; 5′-AGCGCSGGSCGSATCGT-3′) from the amino terminal end of the cyclophilin [33] and the conserved cyclophilin amino acid motif FHRVI (5′-BYCTTCCACCGCGTCATC-3′) were used to amplify an internal fragment of 112 bp. This fragment was used as a probe against a gene library of *S. antibioticus* (provided by Dr. J.A. Salas, Departamento de Biología Funcional, Universidad de Oviedo). Positive colonies were further analyzed and the putative cyclophilin gene identified in the corresponding cosmid. A *Bam*HI fragment of 2.5 kb was subcloned into the *E. coli* pT7T3 plasmid to give the pT7T3-sancyp18. Sequencing was performed with M13 universal primers and completed with specified designed primers (5′-GTGTTCACGCCGGTCG-3′, 5′-CGGCGGGCCGAGACCGT-3′, 5′-CCTCATTCCGGTGGGACTCG-3′, 5′-GGTGGAAAATCGTGCCGTCGT-3′, 5′-ATCAGCGACTTCATGA-3′, 5′-GGCGTAGCCCCAGCCGTTC-3′, 5′-TTCGGCCGGGTCACCGAGG-3′ and 5′-AACCCTTGGCACACTCC-3′). Sequences were processed and analyzed using the GCG programs (Genetics Computer Group).

2.4. SanCyp18 gene overexpression and protein purification

The recombinant pET11a (Novagen)-sancyp18 expression plasmid carrying the *Sancyp18* gene was constructed using the gene-specific primers Sancyp18*Nde*I (5′-GGGAATTCCATATGTCGACAGTCGAGCTGAACA-3′) and Sancyp18*BamH*I (5′-CGCGGATCCGGTCGAGACGGTGCCGCCC-3′), in the appropriate open reading frame to express a native protein without artificial extensions. The recombinant cyclophilin was overexpressed in *E. coli* JM109 (DE3) using standard procedures [45]. Bacteria were ruptured in 15 ml of buffer A containing 0.1 M NaCl (Tris–HCl 20 mM, pH 8.8, EDTA 1 mM, β-mercaptoethanol 7 mM and PMSF 0.5 mM) and ruptured in a MSE soniprep. The cellular extract was applied to a DEAE-Sephacel column (Sigma) equilibrated with buffer A (flow rate 9.56 cm^3/h). Under these conditions, *San*Cyp18 does not bind to the column. The through flow (about 12 ml) was concentrated to a volume of 2 ml using a Centricon 10 (Millipore). The concentrated fraction was applied, in 200 μl volumes, to a Superdex G-75 FPLC column (Amersham Biosciences) equilibrated with buffer A containing 0.15 M NaCl. Fractions containing the pure protein were used to measure cyclophilin activity. Purified *San*Cyp18 were stored at −70 °C.

2.5. Mass spectrometry analysis

Sample was adjusted to 2 pmol/μl with 50% acetonitrile/0.1% TFA. Matrix-assisted laser desorption ionization time-of-flight analysis was performed in a Bruker Ultraflex mass spectrometer (Bremen, Germany) operating in linear mode. DHB was employed as matrix at a concentration of 5 mg/ml in acetonitrile: 0.1% TFA (1:2, v/v) using the dried-droplet method with 1/10 volume of sample. External calibration was performed using a mix of proteins (insulin, ubiquitin I, cytochrome *c* and myoglobin).

2.6. Peptidyl-prolyl cis–trans isomerase (PPIase) and inhibition assays

The substrate specificity (k_{cat}/K_m) of the *San*Cyp18 PPIase was analyzed according to Hani et al. [38] at 10 °C with different peptides as substrates (Bachem, Heidelberg, Germany). The k_{cat} and K_m values were determined in a slightly different manner according to Kofron et al. [39] and calculated by non-linear regression analysis using the Dynafit software [40]. Inhibition of PPIase activity was measured by preincubating CsA with the appropriate *San*Cyp18 enzyme concentration for 5 min at 10 °C before starting the reaction by adding the substrate (succinyl–Ala–Ala–Pro–Phe–NH-Np) and α-chymotrypsin. The inhibition constant (K_i) was calculated with the following equation using SigmaPlot software:

$$f = \frac{k_{enz}}{2} \times ([E] - K_i - [I] + \sqrt{([E] - K_i - [I])^2 + 4[E]K_i})$$

where [E] is enzyme concentration, [I] is inhibitor concentration, and k_{enz} is the enzymatic first-order rate constant determined in the PPIase assay.

IC_{50} value was calculated using the SigmaPlot software, too.

2.7. Isothermal titration calorimetry

CsA binding to *San*Cyp18 was measured by isothermal titration calorimetry (VP-ITC, MicroCal, Northampton, USA) in order to determine stoichiometry, the dissociation constant and thermodynamic parameters of the *San*Cyp18–CsA complex. A *San*Cyp18 solution (1.2 mM) was titrated stepwise into a 50 μM CsA solution. The titration was performed at 10 °C. Before titration, *San*Cyp18 was dialyzed against 25 mM phosphate buffer (pH 7.5), and a 25 mM stock solution of CsA in DMSO was diluted into the dialysis buffer to give the final concentration. The resulting titration curve was analyzed using the manufacturer's software. The characterization of hCyp18 at 10 °C was performed as previously described [41].

2.8. CD spectroscopy

Far UV Circular Dichroism (CD) measurements were performed with a Jasco J-710 CD spectrometer (Gross-Umstadt, Germany). Temperature was controlled in a thermostated cuvette holder by a cryostat RTE111 (Neslab instruments, Portsmouth, USA). An average of eight spectra of *San*Cyp18 were recorded from 180 to 260 nm using a 6 μM protein solution in a 0.1 cm cuvette at 20 °C, the buffer spectrum was subtracted and the molar ellipticity spectra were calculated.

2.9. Viability assay

The viability assay was performed with the LIVE/DEAD L-13152 Bac-Light Bacterial Viability Kit, Molecular Probes, as described previously for submerged *Streptomyces* cultures [42]. The samples were observed under a Bio-Rad MRC600 laser confocal microscope at a wavelength of 488 and 568 nm excitation, and 530 (green) or 630 nm (red) emission.

2.10. Cell fractioning

Protoplasts obtained from *S. antibioticus* GAE liquid cultures [36] were resuspended in buffer A and ruptured as described above for *E. coli*. The unbroken cells and cellular debris were eliminated by centrifugation ($7740 \times g$) at 4 °C for 15 min. Cytosolic membranes were obtained according to Quiros et al. [43] by ultracentrifugation at $75\,000 \times g$ in a Beckman LB-70M ultracentrifuge. The cell-free supernatant fraction was concentrated by 80% ammonium sulfate precipitation. The protein sample obtained after lysozyme digestion was used as the cell wall fraction.

2.11. Analysis of the presence of sancyp18 protein during the developmental cycle of S. antibioticus

The mycelium was grown on the surface of cellophane disks [36]. At different points of time (15, 40 and 96 h), the mycelium was scraped out with a plain spatula, resuspended in buffer A and ruptured in an MSE Soniprep 150. The cellular extracts obtained after centrifuging at 10 000 rpm in an Eppendorf microcentrifuge for 30 min at 4 °C were used for Western blot analysis.

2.12. Immunological methods

Polyclonal antibodies against *San*Cyp18 were obtained by rabbit immunization with the pure protein. After SDS–PAGE, the separated proteins were transferred to PVDF membranes (Immobilon-P Millipore). The membranes were incubated with antibodies for 1 h at room temperature with a serum dilution of 1:1000. Immunodetection was carried out with anti-rabbit-peroxidase IgG (Sigma) using a chemiluminescence reaction of the peroxidase (BM Chemiluminescence Blotting Substrate-POD-Roche).

2.13. Protein analysis

Proteins were analyzed in a 12% gel by SDS–PAGE and stained with silver or Coomassie. Molecular weight markers were from Bio-Rad (Low Range). Determination of protein concentrations was carried out with the Lowry assay [44] using a bovine serum albumin as standard (Sigma). Concentrations of the pure proteins were determined spectrophotometrically; extinction coefficients at 280 nm were calculated from the amino acid sequence according to Gill and von Hippel [45].

2.14. Homology modeling

The modeled three-dimensional structure of *San*Cyp18 was calculated on the basis of its amino acid sequence with the internet-based homology modeling server SWISS-MODEL (http://swissmodel.expasy.org). The "first approach mode" was used with default settings [46–48]. The obtained model was visualized using the SWISS-Pdb-Viewer program.

3. Results

3.1. Analysis of S. antibioticus SanCyp18 cyclophilin gene

The *S. antibioticus* cyclophilin gene was located in a *Bam*HI fragment of 2.5 kb previously subcloned into the *E. coli*pT7T3 plasmid (see Section 2). Sequencing of this fragment showed the ORF of the *San*Cyp18 gene (501 bp). Comparative analysis with streptomycetes promoter sequences [49] allowed the localization of the hypothetical −10 and −35 sequences and the putative ribosome binding site (data not shown). The *S. antibioticus* cyclophilin *San*Cyp18 consists of 166 amino acids and has a predicted molecular mass (M_r) of 18.02 kDa. The G + C content of the coding region is 65%, which is significantly lower than the average 72% deduced from the sequenced genomes of *S. coelicolor* A3(2) and *S. avermitilis* [50,51].

3.2. Purification and characterization of SanCyp18

The *E. coli* strain JM109 harboring the plasmid pET11a-sancyp18 was used to overexpress *San*Cyp18. Purification of the protein was carried out by anionic exchange chromatography and gel filtration, as described in Section 2. The protein elutes from the gel filtration in a single peak with the expected elution volume for 18 kDa proteins. Purified *San*Cyp18 shows a single band with an apparent M_r somewhat higher than the expected in a 12% gel after SDS–PAGE and silver staining (Fig. 1). Purified *San*Cyp18 was analyzed by Far-UV CD-spectroscopy. The recorded spectra are dominated by the signal of large helical content with a minimum at 222 nm, as detected for single domain cyclophilins [16,41]. The molecular mass of the protein was further confirmed by mass spectrometry analysis, from which a 17.9 kDa value was obtained (data not shown).

The substrate specificity of *San*Cyp18 was analyzed spectrophotometrically as previously described [38] using a series of synthetic peptides with the general structure succinyl–Ala–**Xaa**–Pro–Phe–NH-Np, where –Xaa– was substituted by different amino acids (Table 1). Substrate specificity allows discrimination between the different PPIase families. The highest k_{cat}/K_m value for –Xaa– = –Ala– was determined with 7.92 $\mu M^{-1}\,s^{-1}$ (100%) compared to 3.93 $\mu M^{-1}\,s^{-1}$ (49.6%) for –Xaa– = –Phe–,

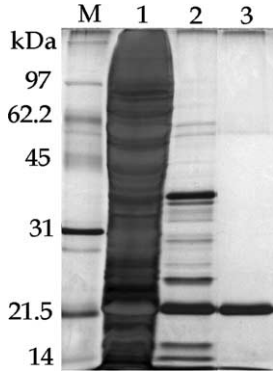

Fig. 1. Purification of recombinant *San*Cyp18. Aliquots from the fractions of the different purification steps were analyzed by silver-staining, after SDS–PAGE (see Section 2). M, molecular weight markers, indicated in kDa (left). Lane 1, crude extract. Lane 2, fraction obtained after DEAE-Sephacel chromatography. Lane 3, fraction obtained after Superdex G-75 FPLC column; the purified *San*Cyp18 is visible as a single band.

Table 1
Specificity of SanCyp18 for different peptide substrates

Substrate –Xaa–	k_{cat}/K_m ($\mu M^{-1}\,s^{-1}$)			
	San Cyp18	Eco CypB[a]	Lp Cyp18	hCyp18[b]
–Ala–	7.92 ± 0.6 (100%)	57.1 (100%)	13.2 ± 0.3 (100%)	17.6 (100%)
–Phe–	3.93 ± 0.5 (49.6%)	14.3 (25%)	6.63 ± 0.3 (50%)	2.7 (15%)
–Arg–	2.12 ± 0.3 (26.8%)	N.D.	3.49 ± 0.3 (26.4%)	N.D.
–Leu–	4.68 ± 0.3 (59%)	27.4 (48%)	7.5 ± 0.29 (56.8%)	5 (29%)
–Gly–	4.9 ± 1 (62%)	21.7 (38%)	7.62 ± 1 (57.7%)	1.8 (10%)
–Glu–	0.54 ± 0.1 (6.8%)	9.1 (16%)	1 ± 0.04 (7.5%)	4.3 (24%)
	K_m (μM)	k_{cat} (s^{-1})	Ref.	
hCyp18	40 ± 50	290 ± 20	Janowski et al. [54]	
*San*Cyp18	181 ± 50	265 ± 22	This work	

The substrates used have the general formulation succinyl–Ala–Xaa–Pro–Phe–NH-Np, where Xaa stands for each amino acid indicated above. Separated K_m and k_{cat} values were determined for the substrate –Xaa– = –Phe–.
N.D. Not determined.
[a] Reported by Compton et al. [16].
[b] Reported by Cavarec et al. [58].

which is a typical substrate specificity for cyclophilins, but different from FKBPs and parvulins [5]. Comparison of the data obtained for four different cyclophilins (*S. antibioticus San*Cyp18, *E. coli Eco*CypB, *L. pneumophila Lp*Cyp18 and hCyp18) showed a clear coincidence between the *S. antibioticus*, *E. coli* and *Legionella* enzymes, but not with hCyp18. Michaelis–Menten parameters K_m and k_{cat} were obtained using the Kofron assay [39] and succinyl–Ala–**Phe**–Pro–Phe–NH-Np as substrate (Table 1). K_m for *San*Cyp18 was determined as 181 ± 50 μM, compared to 40 ± 20 μM for hCyp18. The k_{cat} values for both enzymes are in good agreement. This was calculated as 265 ± 22 s^{-1} for SanCyp18, while the reported value for hCyp18 was 290 ± 20 s^{-1} [41].

3.3. Binding and inhibition by cyclosporine A

The inhibition constants of *San*Cyp18 by CsA were determined as $IC_{50} = 25$ μM and $K_i = 21$ μM by non-linear regression with the given Section 2. The substrate used for this analysis was succinyl–Ala–**Ala**–Pro–Phe–NH-Np (Fig. 2A). Furthermore, the binding of CsA to *San*Cyp18 was determined by ITC. A titration at 10 °C resulted in values for the observed binding enthalpy $\Delta H = -1329 \pm 70$ cal/mol, observed binding entropy $T\Delta S = 4715$ cal/mol and an association constant $K_a = 4.8 \times 10^4 \pm 4.8 \times 10^3$ M ($K_d = 1/K_a = 20.8$ μM). Compared to thermodynamic parameters of hCyp18 binding CsA at the same temperature ($\Delta H = -8341 \pm 35$ cal/mol, $T\Delta S = 2414$ cal/mole, $K_a = 2.0 \times 10^8 \pm 5.8 \times 10^7$ M, $K_d = 5$

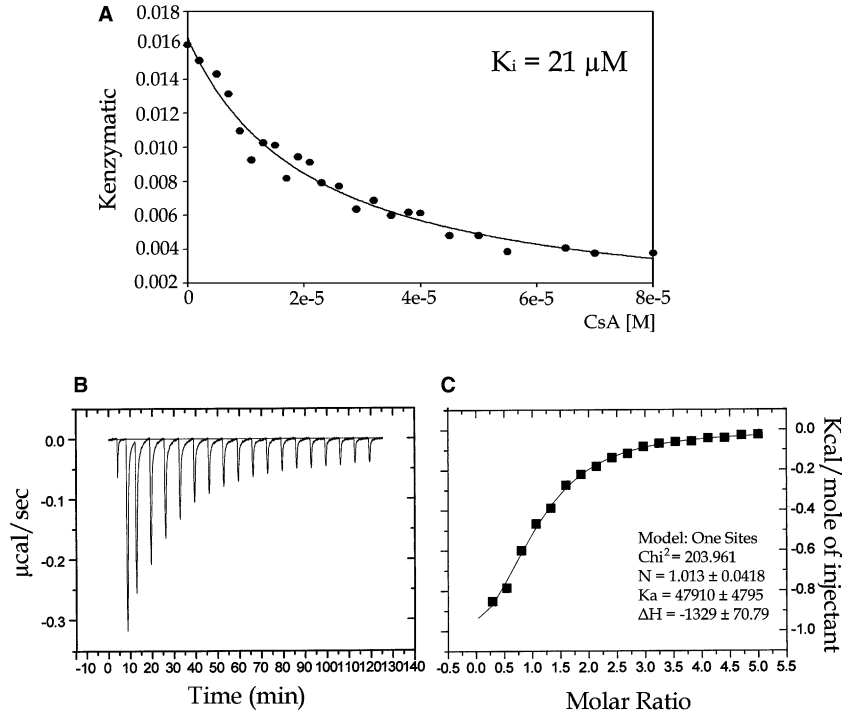

Fig. 2. Determination of the inhibition constant K_i and the dissociation constant K_d. (A) PPIase activity was measured as described using succinyl–Ala–Ala–Pro–Phe–NH-Np as a substrate. The inhibition constant $K_i = 21$ μM was determined as indicated in Section 2. (B) Titration curve of the ITC measurements at 10 °C. A 1.2 mM solution of *San*Cyp18 was titrated stepwise into 50 μM CsA. During titration, the heat evolved as a result of the binding process was recorded over the time. (C) Integrated titration data, baseline corrected and fitted to a one-site binding model. The parameters ($n, \Delta H$ and K_a) for the non-linear regression analysis are shown. The error of each parameter represents the fitting error.

Table 2
CsA sensitivity of cyclophilins from different organisms

Source	Cyclophilin	Identity (%)[a]	IC_{50} nM CsA	K_I nM CsA	Ref.
Human	hCyp18	28.1	9	5	Harrison et al. [56]
Streptomyces chrysomallus (Gram+)	*Sc*CypA	31	25	N.D.	Pahl et al. [23]
Streptomyces chrysomallus (Gram+)	*Sc*CypB	31.5	75	N.D.	Pahl et al. [24]
Bacillus subtilis (Gram+)	*Bsu*CypB	28.17	120	N.D.	Herrler et al. [20]
Streptomyces antibioticus (Gram+)	*San*Cyp18	100	25×10^3	21×10^3	This work
Escherichia coli (Gram−)	*Eco*CypA	59.15	3×10^3	3.4×10^3	Liu et al. [14] and Fejzo et al. [7]
Legionella pneumophila (Gram−)	*Lp*Cyp18	57.32	N.D.	1.2×10^3	Schmidt et al. [26]

N.D. Not determined.
[a] Identity percentage at the protein level between *San*Cyp18 and other cyclophilins.

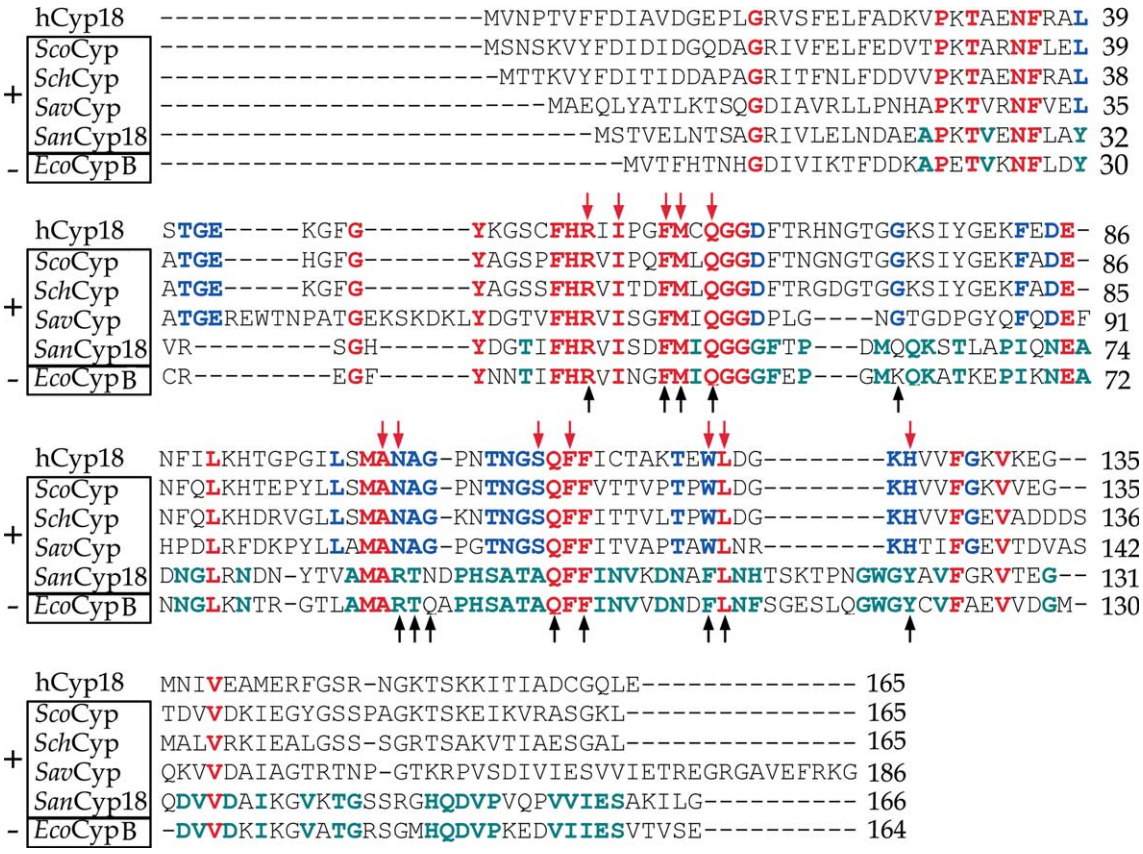

Fig. 3. Sequence alignment of *San*Cyp18 and related cyclophilins. The sequences included are hCyp18 (Accession No. P05092), cyclophilins from Gram positive bacteria (*Sco*Cyp, *S. coelicolor* A3(2), Accession No. CAC42137, *Sc*CypA, *S. chrysomallus*, Accession No. S28020, *Sav*Cyp, *S. avermitilis*, Accession No. NP_825506.1, *San*Cyp18, Accession No. AY343890 and a representative cyclophilin from Gram negative bacteria (*Eco*CypB, *E.coli* Accession No. P23869). Amino acid residues involved in CsA (black arrows) and substrate (red arrows) binding of hCyp18 [3,41,54] are indicated. Amino acids conserved among cyclophilins from Gram positive and hCyp18, are in blue; those conserved among cyclophilins from Gram negative bacteria and *San*Cyp18 are in green; and those conserved in all the cyclophilins are in red.

nM), $\Delta H_{SanCyp18}$ is raised >6-fold and $T\Delta S_{SanCyp18}$ is doubled. The determined *San*Cyp18 K_d by ITC and the kinetically obtained K_i value are in good agreement (Fig. 2B and C).

*San*Cyp18 binds weakly to CsA compared to hCyp18 and other cyclophilins of Gram positive bacteria, including several *Streptomyces* species and *Bacillus* (Table 2). The IC_{50}, K_i and K_d values for the inhibition of *San*Cyp18 by CsA are in the lower micromolar range, which is similar to the ranges published from Gram negative bacteria cyclophilins (as *E. coli* and *L. pneumophila*; Table 2).

3.4. Structural homology of SanCyp18 and Gram negative cyclophilins

The sequence of *San*Cyp18 cyclophilin was compared to those from other *Streptomyces* and Gram negative bacteria available in the databases. Fig. 3 shows the amino acid sequence alignment of *San*Cyp18 and representative cyclophilins from *Streptomyces* and Gram negative bacteria. Clearly, *San*Cyp18 shows a significant higher homology to the latter, represented by *E. coli Eco*CypB cyclophilin. To further support this, a model of the three-dimensional structure of *San*Cyp18 was obtained as described in Section 2. Five (2NUL, 1LOPa, 1CLH, 1C5Fc and 1C5Fa) out of >50 homologous cyclophilin structures were selected from the protein data bank (PDB) by the program to calculate the *San*Cyp18 model (Fig. 4A). The resulting model has a root mean square deviation of 0.48 Å calculated for the backbone superposition with 1LOPa. This gives the X-ray structure of *Eco*CypB complexed with the peptide

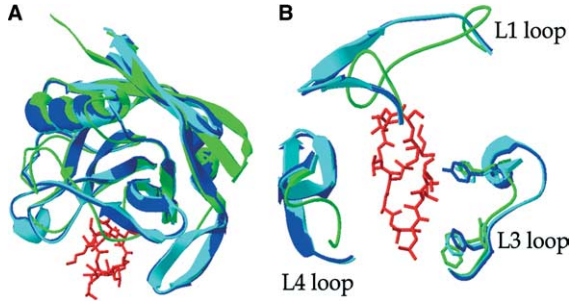

Fig. 4. Three-dimensional structure comparison between hCyp18, *Eco*CypB and *San*Cyp18. (A) Model of the three-dimensional structure of *San*Cyp18 superposed to *Eco*CypB (PDB: 1LOPa) and hCyp18 (PDB: 1CWA). The backbone structures are presented as ribbons, indicating secondary structure elements (loops, helices and β-sheets). Rmsd values for backbone superposition are 0.48 and 0.9 Å for *San*Cyp18 with *Eco*CypB and hCyp18, respectively. Green: hCyp18, light-blue: *Eco*CypB, dark-blue: *San*Cyp18, red: CsA. (B) Magnification of three loop regions that exhibit the largest structural differences between hCyp18 and *Eco*CypB/*San*Cyp18. Loop caption is according to [51]. Tryptophan 121 and Histidine 125 (hCyp18) and the respective amino acids are indicated.

succinyl-Ala-Pro-Ala-NH-Np [52]. The calculated rmsd for the backbone superposition with the structure 1CWA, representing the X-ray structure of hCyp18 complexed with CsA, is 0.97 Å [26]. The overall backbone superposition presents a good fit with the general structure of cyclophilins, especially for most secondary structure elements of α-helices and β-sheets. Some differences are obvious in loop regions, compared with the structure of hCyp18 (1CWA) (Fig. 4B). As described also for *Eco*CypB, three loops (*San*Cyp18 amino acids 55–70; 105–122; 140–156) vary in their three-dimensional orientation and size [52].

3.5. Cell location and presence of SanCyp18 on differentiating cultures

The location of *San*Cyp18 was analyzed with cultures grown in liquid GAE supplemented with 0.5% glycine cultures by Western blot reactions with *San*Cyp18 polyclonal antibodies (see Section 2). The viability of the cells used for this analysis was tested as described previously [42]. In cultures grown for 24 h, all cells were in a viable state (Fig. 5A). *San*Cyp18 was not detected in the cell-free supernatant obtained from the culture medium (Fig. 5B). Thus, the presence of *San*Cyp18 in the cytoplasm, cell membrane and cell wall was investigated after fractionation of protoplasts. *San*Cyp18 was found within the cytoplasm (Fig. 5B) but not in the membrane and cell wall fractions, however some membrane-located, high molecular weight proteins gave a cross-reaction with the *San*Cyp18 antibody (Fig. 5B).

The presence of *San*Cyp18 was analyzed during the developmental cycle of *S. antibioticus* at the three representative steps of development in surface cultures: substrate, aerial and sporulating aerial mycelium. As shown in Fig. 5C, the protein is detected in all three phases, suggesting that it is constitutively expressed.

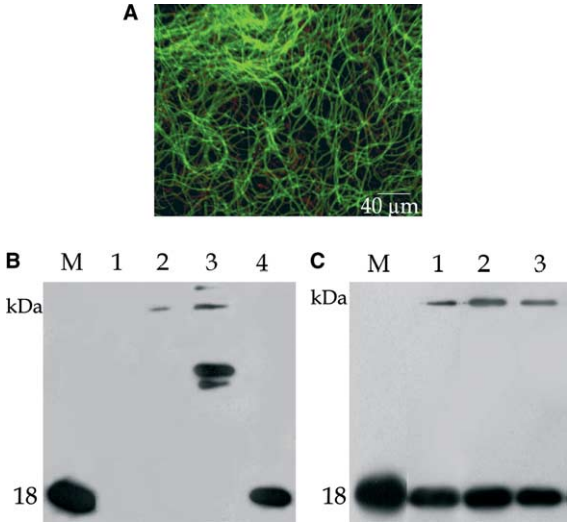

Fig. 5. Cell location and presence of *Streptomyces antibioticus* cyclophilin in different developmental stages. (A) Viable state of the cells (24 h of culture) tested with the LIVE/DEAD Bac-Light Bacterial Viability Kit. Mycelium appears green, which is an indicator of viability (see Section 2). (B) Western blot analysis of the different cell fractions probed with the *San*Cyp18 polyclonal antibodies (see Section 2). Lane M, marker (1 µg of purified recombinant *San*Cyp18); Lane 1, cell wall fraction; Lane 2, cell-free culture medium; Lane 3, membrane fraction; Lane 4, cytoplasmic fraction. All fractions contain 50 µg of protein. (C) Western blot analysis of the presence of *San*Cyp18 during the cell-cycle of *S. antibioticus*; M, marker (1 µg of purified recombinant *San*Cyp18); 1, substrate mycelium (about 15 h); 2, aerial mycelium (40 h); 3, sporulating aerial mycelium (96 h). All fractions contain 50 µg of protein.

4. Discussion

The first PPIases of the cyclophilin type characterized in *Streptomyces* were those of *S. chrysomallus*, named cyclophilin A (*Sc*CypA) and cyclophilin B (*Sc*CypB) [23,24]. These cytosolic proteins, of 17.5 and 18.8 kDa, show sequence homology to eukaryotic hCyp18 and exhibit similar CsA sensitivity. Furthermore, they are noticeably different from cyclophilins from Gram negative prokaryotes and the cyclophilin described in this paper (Fig. 3) [23,24]. The physiological role of these cyclophilins, as those from other organisms, remains unclear [3]. High-level expression of *Sc*CypA and *Sc*CypB did not produce detectable changes in the growth or morphology of *S. chrysomallus* [24].

The *S. antibioticus* cytosolic cyclophilin characterized in this work was proposed in a former report to be also a nuclease [33], as analogously reported for NUC-18, a thymocyte nuclease which was found to share amino terminal end sequence homology with proteins belonging to the cyclophilin family and which was proposed to play the key role in glucocorticoid-stimulated apoptosis [34]. However, an analysis of the *San*Cyp18 recombinant protein has shown the absence of such cyclophilin-associated nuclease activity [53].

One function of PPIases is related to the correct folding of proteins. Together with the disulfide-isomerases (EC 5.3.4.1.) and the secondary amide bonds *cis*/*trans* isomerases (APIases), they form the group of folding helper enzymes [54]. To analyze whether *San*Cyp18 exhibits the functional characteristics of a cyclophilin, we compared the enzymatic properties of purified *San*Cyp18 with other previously characterized cyclophilins from prokaryotes and eukaryotes. We showed that the purified protein exhibits a CD-spectrum with high similarity to CD-spectra of other single domain cyclophilins such as hCyp18. Comparison of the determined *San*Cyp18 substrate specificity with the substrate specificities of *Eco*CypB, *Lp*Cyp18 and hCyp18 revealed that there is a higher similarity between the three bacterial cyclophilins with respect to hCyp18 (Table 1). This is in accordance with the homology observed at the sequence level (Fig. 3) and the homology modeling approach (Fig. 4). Michaelis–Menten parameters obtained for *San*Cyp18 are in the same range as that known for hCyp18 (Table 1). Accordingly, it may be stated that *San*Cyp18 shows enzymatic activities that are characteristic of cyclophilins.

The CsA inhibition constant K_i for *San*Cyp18 is much higher in comparison to that for hCyp18 and the inhibition constants for cyclophilins from Gram positive bacteria (Table 2). *San*Cyp18 is the only cyclophilin characterized to date in a Gram positive bacterium that shows an inhibition constant for CsA in the micromolar range ($K_i = 21$ µM). This is a value that is characteristic of cyclophilins from Gram negative bacteria. Other Gram positive bacteria and eukaryotic cyclophilins have K_i and IC_{50} values in the nanomolar range (Table 2). This can be explained by the fact that cyclophilins from Gram negative bacteria and *San*Cyp18 differ from hCyp18 in two aspects. Firstly, there are three regions in the *Eco*CypB crystal structure (1LOPa) and the calculated *San*Cyp18 model that show a large

deviation in their loop arrangements. The corresponding regions in the hCyp18 crystal structure 1CWA are amino acids 65–82, 117–126 and 147–155. The formed loops are necessary to build up contacts with CsA (Fig. 4) [55]. Secondly, *San*Cyp18 and cyclophilins from Gram negative bacteria have changed five of the thirteen particular amino acids that intervene in CsA binding [18] (Fig. 3). The most relevant amino acid changes are histidine 125 and tryptophan 121 (both hCyp18 numeration), which are essential for CsA binding and consequently for PPIase activity inhibition. An intermolecular hydrogen bond is formed from the side chain of this tryptophan with the CsA molecule [14]. The corresponding amino acid in *Eco*CypB is phenylalanine 112 (Fig. 3). A substitution of this phenylalanine 112 to tryptophan increases the affinity of *Eco*CypB to CsA 20-fold (from 3.4 μM to 170 nM), whereas the exchange of hCyp18 tryptophan 121 to phenylalanine reduces its affinity 20-fold (17–490 nM) [56]. The corresponding amino acid of *San*Cyp18 is also a phenylalanine (Fig. 3). The calculated binding enthalpy $\Delta H_{\mathrm{SanCyp18}}$ derived from the ITC experiments is less negative in comparison to that of $\Delta H_{\mathrm{hCyp18}}$. This might be explained by the lack of the intermolecular hydrogen bond between cyclophilin and CsA resulting from the missing tryptophan. This results in the higher binding enthalpy determined for the binding of CsA to *San*Cyp18.

A complete analysis of *Streptomyces* cyclophilins has shown eukaryotic-type cyclophilins similar to those mentioned above in *S. chrysomallus* [24], in several *Streptomyces* species, including *S. coelicolor* (Manteca et al., submitted). Interestingly also, a few *Streptomyces* species harbor cyclophilins with similar characteristics to those of *San*Cyp18 analyzed here (Manteca et al., submitted). One of them, from *S. achromogenes*, is almost identical (92.6% identity) to *San*Cyp18. The presence of these Gram negative-like cyclophilins could be explained by horizontal gene transmission (HGT) of the gene from this group of bacteria to the above mentioned species. In fact, HGT seems specially relevant within this group of bacteria, as previously reported by other authors [57].

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